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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Craig L.	Nessler	Blacksburg, Va.

☐ *Additional inventors are being named on the____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

A NOVEL METHOD FOR REDUCING TOBACCO SPECIFIC . NITROSAMINES (TSNAs) IN AIR-CURED VARIETIES

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☐ Application Data Sheet. See 37 CFR 1.76

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☒ Applicant claims small entity status. See 37 CFR 1.27.

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Respectfully submitted,

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Date 12/18/02

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Docket Number: 01640289P2

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P18SMALL/REV05

A NOVEL METHOD FOR REDUCING TOBACCO SPECIFIC NITROSAMINES (TSNAs) IN AIR-CURED TOBACCO VARIETIES

Tobacco specific nitrosamines (TSNAs) have been virtually eliminated from flue-cured tobacco by modifying the curing barns to separate drying tobacco leaves from the combustion gases produced by conventional heaters. Unfortunately, TSNA levels have not been significantly reduced in air-cured tobacco due to microbial activity and the high costs associated with eliminating microbes from the leaf source. I have invented a low cost method for reducing TSNA accumulation in air-cured tobacco through a novel approach of scavaging nitrogen oxides during air-curing. This invention could have significant impact on the tobacco industry and the public health by substantially reducing a major class of potent carcinogenic compounds, TSNAs, in air cured tobacco.

TSNAs are highly carcinogenic compounds formed from the nitrosation of tobacco alkaloids during curing. Current evidence suggests that TSNAs in flue-cured tobacco are formed from the direct interactions of combustion products with the leaf alkaloids during the curing process. A $57 million dollar program has just been completed by the major U.S. tobacco companies to convert domestic curing barns so that combustion gasses can be vented from barns instead of mixing with the drying leaves. While this conversion appears to have eliminated TSNAs from flue-cured tobacco, no simple engineering fix is currently available for air-cured varieties. In the case of these varieties, TSNA accumulation appears to result from microbial activity during the prolonged curing process in open air. Experiments reported at the 55th Tobacco Science Research Conference (10 September 2001) indicate that infiltration of air-cured tobacco leaves with ascorbic acid substantially reduces the accumulation of TSNAs (see Appendix 1).

In previous work, unrelated to TSNAs, we have shown that ascorbic acid levels can be increased in *Nicotiana tabacum* cv. Xanthi, an air-cured tobacco variety by expressing the gene encoding L-gulono-gamma-lactone oxidase (GLOase) the terminal enzyme in the animal ascorbic acid biosynthetic pathway. The invention is therefore to use this approach to reduce TSNAs in commercially produced tobacco.

In short, work by other groups has shown that infiltration of ascorbic acid into air-cured tobacco leaves greatly decreases the accumulation of highly carcinogenic TSNAs. Work in my laboratory (see Appendix 2) has shown that internal ascorbic acid levels can be increased up to 7-fold in tobacco leaves by expressing a rat gene encoding L-gulon-gamma-lactone oxidase (GLOase) there terminal enzyme in the animal ascorbic biosynthetic pathway. Therefore, my invention would be to use the GLOase gene or other genes in the ascorbic acid biosynthic pathway for the purpose of reducing TSNA accumulation in air-cured tobacco by increasing their ascorbic acid levels during curing. Additional improvement would include the use of desiccation and senescence induced promoters that would increase expression of ascorbate pathway genes in tobacco leaves as they dried and yellowed during the air-curing process.

Expression of L-gulono-γ-lactone oxidase (GLOase) in burley 509 increases ascorbic acid levels

Principal Investigator: Craig L. Nessler

Experimental Results: Eighteen burley 509 tobacco lines expressing a rat cDNA encoding L-gulono-γ-lactone oxidase (GLOase) were created using *Agrobacterium tumefaciens* mediated transformation. Burley 509 was chosen as a target for metabolic engineering because it is an air-cured commercial variety particularly prone to developing tobacco specific nitroamines (TSNAs) during the curing process. Thus 509 represents an ideal target for examining the phenomenon of TSNA reduction resulting from the endogenous production of ascorbic acid.

Analysis of the GLOase over expressing plants showed 2-8 fold increases in ascorbic acid compared to wild-type plants. Data from this analysis is shown in table 1 and presented graphically in figure 1.

Ongoing Research: Experiments are underway to determine the TSNA levels in nine of these lines currently being air-cured in a tobacco barn at our Glade Spring Agricultural Research and Extension Center (AREC). The GLOase expressing 509 stalks taken from greenhouse grown plants were placed in the middle of nontransgenic field grown stalks.

Field experiments will begin during the spring of 2003. Plants will be grown from seed in the Virginia Tech greenhouses and 4-6 week old seedlings will be taken to the Blackstone and Glade Spring ARECs for hardening and transplanting to the field. The field plots will consist of 16 plant rows in a randomized complete block design.

Both engineered and control plants will be grown using standard burley production methods and although no extraordinary measures will be taken in their cultivation they will be treated for diseases or insect infestations as needed. The overall agronomic performance of the experimental and control plants will be observed throughout the growing season and any unusual features that might be due to elevated ascorbic acid levels noted.

Experimental and control tobacco plants will be harvested at the end of the growing season and cured in open barns at the stations along with other field grown material to maximize exposure of the leaf to microorganisms that may be associated with TSNA production during the cure. Following the curing process leaf will be subjective to extensive chemical profiling including TSNA analysis.

Table 1: Ascorbic Acid Levels in GLOase 509 Burly Tobacco

Plant #	Date planted	Ascorbic acid content µM/g of Fresh tissue
1	05/31/02	3.042
2	06/18/02	4.420
3	05/31/02	3.548
4	06/18/02	2.656
5	07/02/02	4.532
6	07/02/02	2.762
7	07/08/02	4.318
8	07/08/02	4.405
9	07/19/02	3.328
10	07/19/02	2.446
11	07/19/02	6.276
12	07/19/02	2.902
13	07/28/02	3.496
14	07/19/02	8.304
15	07/07/02	2.237
16	07/19/02	5.817
17	07/07/02	3.351
18	07/19/02	2.657

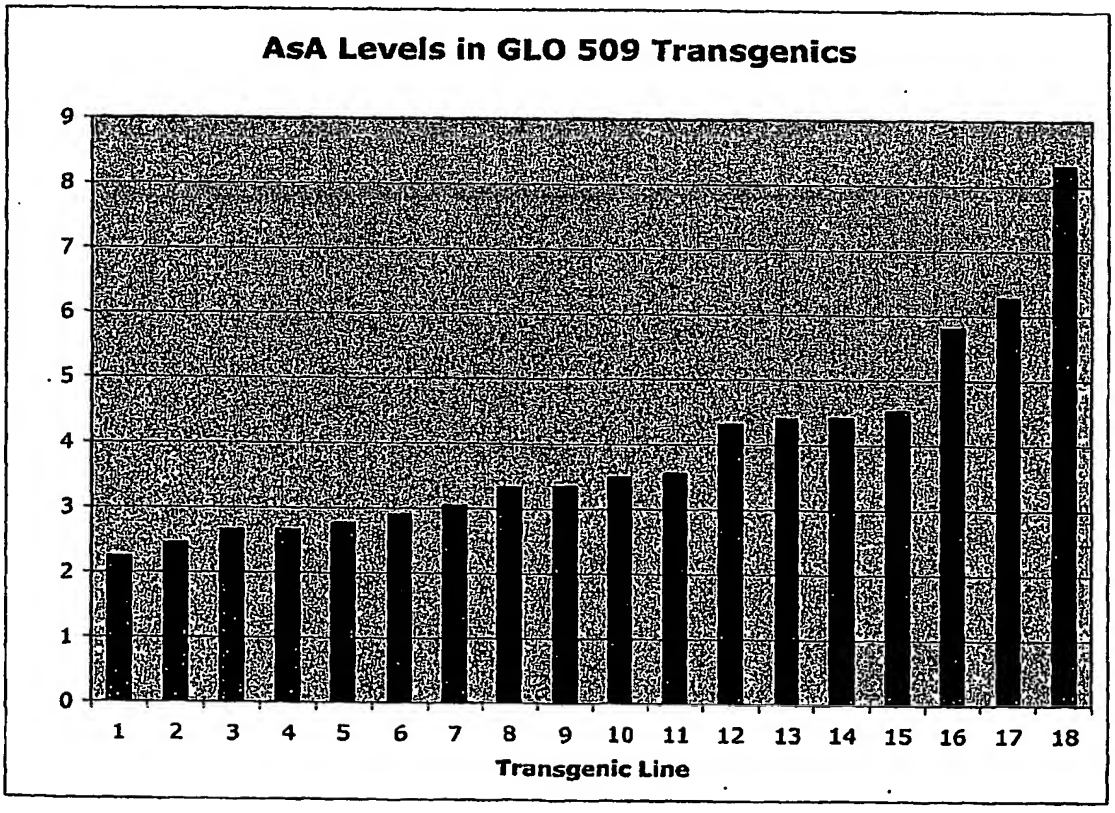

Figure 1: Ascorbic Acid Levels in GLOase 509 Burly Tobacco

CONCLUSION

Three hundred and seventy-five tobacco samples were obtained from both direct-fired and indirect-fired curing barns in the five flue-cured tobacco producing states during the 2000 curing season. Analysis of these samples have shown conclusively that curing tobacco in the absence of combustion gases reduces TSNA levels in the cured leaf by more than 93 percent. Although other factors may have a very minor effect on TSNA levels in cured leaf, the single greatest reduction in levels occurred when the products of curing fuel combustion were kept from the curing air. With lessons learned in 2000, we believe that it may be possible and practical to have, within several years, an overall average TSNA level in US flue-cured tobacco of 0.20 ppm or less.

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APPENDIX I

FORMATION OF TOBACCO-SPECIFIC NITROSAMINES IN AIR-CURED TOBACCO

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ABSTRACT

Tobacco-specific N'-nitrosamines (TSNA) are formed during curing and are derived from the reaction of a nitrosating species with the tobacco alkaloids. Most important of these reactants are nitrite and the secondary amine alkaloids. Nitrogen fertilizer increases the amount of alkaloids and nitrate that will accumulate in the plant. Nitrite does not accumulate in plants and the source of nitrite formation during the air-curing process most likely is microbial, but nitrite accumulation is not correlated to the amount of nitrate present in the leaf. Nitrite scavengers do decrease TSNA formation, however no good application or cultural practice is known to increase the levels of nitrite scavengers in the leaf at the critical time during the curing process. Modification of the plant or the curing environment to increase the rate of drying during the curing process decreases TSNA accumulation in the cured leaf. However, none of these techniques has resulted in a consistently high quality useable leaf with low TSNA concentration. Greatest immediate reduction in TSNA accumulation may be achieved by reducing the secondary amine alkaloids in the tobacco line grown.

INTRODUCTION

Nitrosamines have been known for nearly 150 years but it was not until about 50 years ago that they became the subject of much investigation. The main reason for the increased interest was the toxicity of many of these compounds. N'-Nitrosamines are present in food, cosmetics and tobacco products and are an important group of organ specific tumor inducers in tests with laboratory animals (Chung et al, 1994; US Surgeon General, 1982, 1989). Some nitrosamines of tobacco and tobacco smoke have received particular attention since they are found only in tobacco and have biological activity. The unique N'-nitroso compounds in tobacco are N'-nitroso derivatives of tobacco alkaloids and are called tobacco-specific N'-nitrosamines (TSNA). Formation of TSNA is primarily a post-harvest event in both flue-cured and air-cured tobacco. Wiernik et al (1995) summarized available research results and formulated the hypothesis that

the nitrite in the tobacco responsible for TSNA formation derives from microbial activity. It is the intention in this review to present new evidence that support this hypothesis and to discuss potential techniques and modifications to the current curing process that may lead to reduction of the TSNA accumulation in air-cured tobacco. The reader is referred to previous citations in *Recent Advances in Tobacco Science* for more detailed information on the physical and chemical changes that occur during air-curing (Burton et al, 1983, Walton et al, 1995; Wiernik et al, 1995).

GENERAL THEORY OF FORMATION

Tertiary and secondary amine alkaloids of tobacco are nitrosated to form TSNA. In solution at low pH, nitrosation of nicotine yields the nitrosamines NNA [4-(-methylnitrosamino)-4-(3-pyridyl)-1-butanal], NNN (nitrosonornicotine) and NNK [4-N-methylnitrosamino)-1-(3-pyridyl)-1-butanone]. Nitrosation of nicotine is first order with respect to both nicotine and nitrite, the nitrosating species (Caldwell et al, 1991). Nicotine nitrosation is a reversible reaction and the rate-determining step. The intermediate undergoes a slow elimination of HNO to yield an iminium ion. The reactions of nicotine with a nitrosating species follow the general equation of Scheme 1.

Scheme 1

$R_3NNO^+ <-> k[\text{nicotine}][NO_2^-]$

$R_3NNO^+ -> R_2N{=}CHR^+ + HNO$

Scheme 1 does not readily apply to a tobacco leaf because of the more neutral pH and low water content, therefore nitrosation in situ occurs preferentially with the secondary amines. Formation of nitrosamines from secondary amine alkaloids - nornicotine, anabasine, anatabine and pseudo-oxynicotine (PON) - is first order for alkaloid concentration and second order for nitrite (Scheme 2). The rate-limiting step is the initial nitrosation step.

Scheme 2

$R_2NNO = k[R_2NH][NO_2^-]^2$

Usually the alkaloids are found in much greater amounts than nitrite in tobacco and thus the significance of microbial production of nitrite during curing is highly significant for TSNA accumulation. However, under certain conditions the alkaloids may be the rate-limiting precursor.

CURRENT HYPOTHESIS ON THE FORMATION OF TSNA DURING AIR-CURING

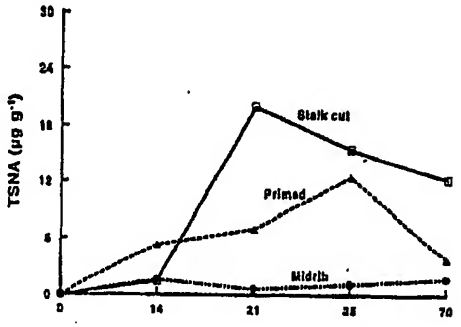
TSNA are present at very low levels in green tobacco during the vegetative growth stage. At topping when the plant is going into the reproductive stage, low levels are more readily detected and by harvest time 20 to 100 ng g^{-1} are often measured. In the early stages of air-curing little change in TSNA levels is measured, but after

the end-of-yellowing stage and before the midrib is dried significant amou[redacted] TSNA may accumulate (Fig. 1). In Kentucky, USA, this accumulation freq[redacted] occurs between days 14 and 30 of the curing process and is dependent upon the curing environment.

These observations have been rationalized in terms of loss of cellular integrity due to hydrolysis and moisture loss by the end-of-yellowing. The cell content leaks into the intracellular spaces and is made available as substrate for bacteria. Some of these bacteria, e.g. *Enterobacter agglomerans*, have a dissimilatory nitrate metabolism and reduce nitrate with subsequent accumulation of nitrite. The nitrite formed reacts with the tobacco alkaloids and TSNA are produced. As the curing proceeds, the water content of the tobacco leaf decreases and the water activity gets too low for continued bacterial activity. Support for this hypothesis has come from surveys on bacterial populations on the tobacco leaf during curing, from measurements of nitrate reductase activity (NRA) in the tobacco plant and on the tobacco leaf during curing. In addition, the accumulation of nitrite and TSNA during the course of the curing has been monitored in several studies. Other studies have shown the effect on TSNA accumulation by intervention of a nitrite scavenger such as ascorbic acid and by inactivation of microbial growth via rapid drying of the tobacco before the critical stage of nitrite formation (i.e. end-of-yellowing).

Because the TSNA formation in air-cured tobacco is highly dependent upon the ambient conditions, it is understandable that the levels of TSNA vary significantly from year to year and from country to country. Thus, samples from experimental trials in Kentucky each year have shown TSNA levels ranging from 1.4 to 89 μg g^{-1} (Table 1). These data do not represent the TSNA accumulation in commercial tobacco but illustrate the range that frequently will be found in cured leaf. NNN is often present in greatest amounts but on occasions NAT or NNK will be present in greatest amount.

Figure 1. Total TSNA accumulation in the midrib of primed leaf, midrib only and stalk-cured control during air-curing of KY 171 dark tobacco in 1996.



In a normal to wet curing season midrib tissue accumulates greater amounts of TSNA than lamina. The reverse may be true for a dry curing season. This difference may be due to the fact that during a wet season comparatively higher levels of nitrite are found in the midrib than in lamina. During a dry season the levels of nitrite are low both in lamina and midrib and the higher levels of alkaloids in lamina are reflected in higher TSNA levels.

Table 1. Range of Nitrosamines in Burley Tobacco Air-Cured in Kentucky

Nitrosamine	Lamina (µg/g)	Midrib (µg/g)
NNN	0.41-18	0.43-43
NAT	1.20-13	0.80-25
NNK	0.09-07	0.15-22
TSNA	1.70-38	1.38-89

NEW EVIDENCE THAT SUPPORT THE CURRENT HYPOTHESIS

1. Bacteria

Greenhouse-grown tobacco usually has very low TSNA levels in the cured leaf, and Cui (1998) proposed that this was because of the lack of bacterial contamination associated with the leaf. Greenhouse-cultured tobacco treated with a dilute filtered, soil suspension prior to harvest had increased in vitro NRA and nitrite levels between days 14 and 28 of the air-curing. TSNA formation was positively correlated to the nitrite increase. TSNA levels in the greenhouse-grown tobacco were equivalent to those from field-grown tobacco. These results suggest the importance of soil-borne microbes in the formation of TSNA during the air-curing process.

Steele and Hempfling (2000) reported that during the air-curing process of burley tobacco the bacterial populations increased from 104 to 106 per gram and confirmed that *Enterobacter spp.* were the most abundant bacteria throughout the curing process. The presence of abundant *Enterobacter spp.* is significant in that many *Enterobacter spp.* are known to reduce nitrate and accumulate nitrite, and their presence during the time of TSNA accumulation is most likely a cause and effect relationship (Wiernik et al, 1995).

Lei (2000) found that there were no differences in numbers of bacteria colony-forming units isolated from eight different tobacco lines (including burley, bright, dark and low alkaloid genotypes) at each sampling period from topping to cured leaf. In 1999, bacteria populations increased from the time of topping to the cured leaf. However, in 2000, the numbers were similar throughout the sampling

period. The curing season in 1999 was unusually warm and dry and the numbers were lower than in 2000, which was a normal curing season.

2. Nitrate Reductase Activity (NRA)

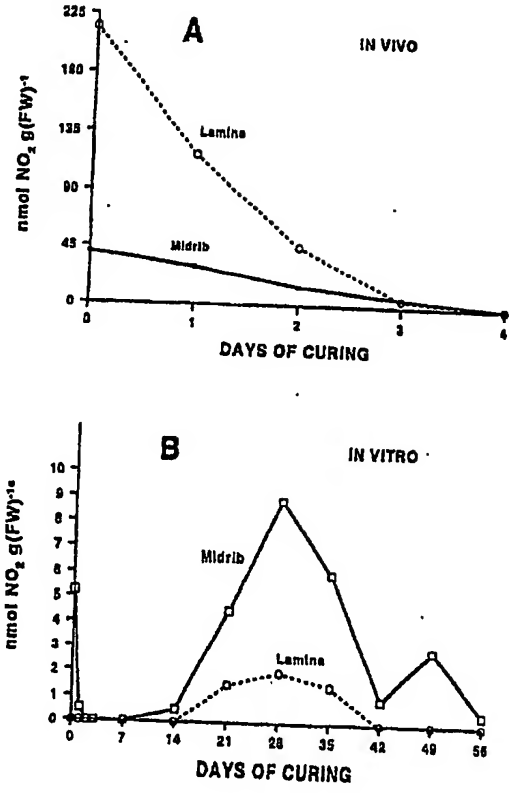
In the green plant, nitrate is reduced to nitrite by the enzyme nitrate reductase (NR). This is the first of two enzymes in assimilation of nitrate into ammonium used in the biosynthesis of amino acids and other reduced nitrogen forms in the plant. The second step is the reduction of nitrite to ammonium by the enzyme nitrite reductase. These reactions are known as assimilatory nitrate reduction. Nitrite does not accumulate in green tissue and plant NRA is rapidly lost upon harvest possibly due to water stress. Bacteria and fungi have NRA that can be grouped into assimilatory and dissimilatory activity. Assimilatory activity is present under aerobic conditions and is similar to the plant NR in that ammonium and thus reduced nitrogen forms are available to the organism. In dissimilatory nitrate reduction, NO_3^- acts as the terminal oxygen acceptor under anaerobic conditions and the NO_3^- is reduced to NO_2^-, which may accumulate. No further reduction occurs unless it proceeds to nitrous oxide or N_2 in a series of steps called denitrification. Because plant NR is highly sensitive to water stress, any accumulation of nitrite during curing is hypothesized not to come from plant NRA but from microbial NRA. This conclusion has now received additional experimental support.

In a series of experiments that measured in vivo and in vitro NRA in the leaf during air-curing, Cui (1998) measured NRA of 650 nmol NO_2^- g^{-1} (fw) for lamina and 150 nmol NO_2^- g^{-1} for midrib in 1994. Only about one-third of the activity detected at topping was observed at harvest, day 0 of curing (Fig. 2A). Leaf NRA sharply decreased after harvest and was near zero at day 3 and was not detected after day 4 regardless of tissues. Leaf moisture loss in the first three days of curing was only 10 percentage units for lamina and 5 percentage units for midrib, statistically non-significant, but resulted in over 95% loss of NRA. This again, supports the hypothesis that plant NRA is very sensitive to water stress and probably not functional after a few days of air-curing. Much lower NRA was observed in midrib than lamina. NRA in lamina measured with the universal electron donor, methyl viologen, was slightly higher than that measured with NADH, a plant specific electron donor. This observation suggests the presence of microbes on the leaf surface contributing to NRA.

In vitro leaf NRA (Fig. 2B) was lower compared to in vivo NRA. In vitro activity was present at harvest but decreased below detectable level in 1 or 2 days after harvest and was again detectable after 2 weeks. There was a very significant increase at week 3 and maximum activity was measured at week 4. NRA was most pronounced in midrib with the universal electron donor methyl viologen. NRA in lamina with NADH as electron donor was low and disappeared early.

At week 3, the rapid moisture loss and the significant increase in conductivity of leaf cell leachate (Fig. 3) marked the degradation of leaf cell membranes which would suggest an increased protein degradation and loss of organelle compartmentation, resulting in an altered leaf NR system or the existence of another NR system associated with these plants (Bush, 1981; Bush et al, 1993; Cram, 1973; Ferrari et al, 1973; Beevers, 1965; Tso, 1990). These results (Fig. 2) suggest that NRA detected after week 3 was not plant NR. At the same time, the loss of leaf membrane integrity made all nutrients available and provided an opportunity for microbe invasion. The favorable temperature, relative humidity and partial lack of oxygen supply in the curing barn (Burton et al, 1989b; Hamilton et al, 1982a) certainly will support microbial activity in and on the tobacco matrix. Theroretically, because midribs retain more moisture and have greatest nitrate level (Mosely et al, 1961; Broaddus et al, 1965; Burton et al, 1983, 1989b, 1992), there should be a higher NRA in the midrib than lamina if microbes are present, an observation supported by the data in Fig. 4.

Figure 2. A. In vivo and B. in vitro NRA in lamina and midrib during air-curing of burley tobacco in 1994.



In this experiment, the lamina nitrite concentration remained reasonably constant at about 1 μg g^{-1} fw from harvest to the end of curing (Fig. 4). Midrib nitrite usually is not detectable at harvest, and does not significantly change from day 1 to day 14 of curing, though it slowly increased from 0.3 to 1μg g^{-1} in this experiment. However, nitrite concentration was significantly increased during week 3 and week 4, and remained at about 4 μg g^{-1} during the later stages of curing. Nitrite accumulation during the third week of curing has been reported also by Burton et al, 1989b; Bush et al, 1995; and Qi, 1991.

During the three years of the experiment, in which two cultivars were used, there was always a significant and positive relationship between in vitro NRA and nitrite accumulation, and between nitrite accumulation and TSNA accumulation regardless of year, cultivar or curing conditions. Since alkaloids and nitrite are direct precursors for TSNA formation and the alkaloids are already in the matrix, nitrite accumulation probably directly resulted in the subsequent TSNA formation.

Figure 3. Leaf moisture and conductivity during air-curing of burley tobacco in 1994.

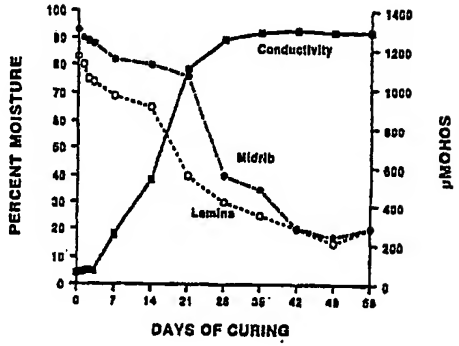
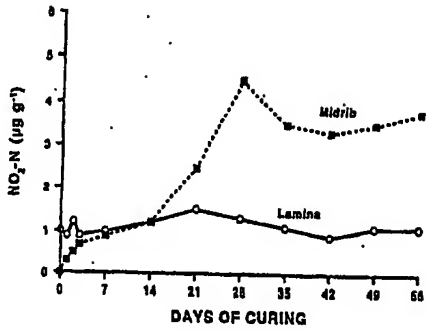


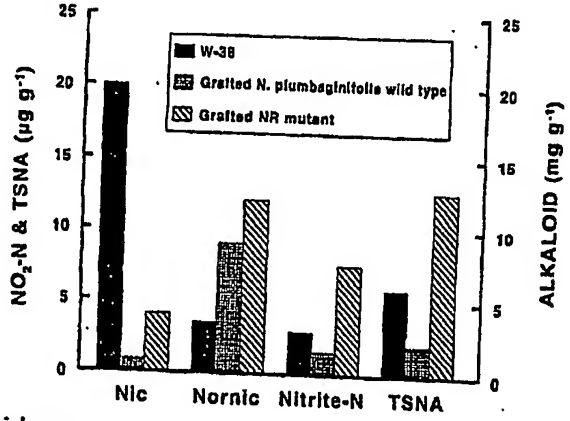
Figure 4. Nitrite accumulation in lamina and midrib during air-curing of burley tobacco.



These results support the concept that plant in vivo NRA decreases rapidly after harvest and that plant NRA does not result in detectable nitrite accumulation. They also provide strong support for the hypothesis involving microbial reduction of nitrate to nitrite during the time of cellular disruption.

Additional strong support for the significance of microbial NRA for nitrite formation and subsequent TSNA formation was obtained by grafting a NRA deficient mutant of *N. plumbaginifolia* onto *N. tabacum* cultivar Wisconsin 38 (Cui, 1998). The mutant was a nia gene deficient mutant that has no mRNA expression, thus no nitrate reductase enzyme in the plant. NRA was not detectable in the mutant but was detectable at a rate in the wild type *N. plumbaginifolia* equivalent to the nongrafted wild type and Wisconsin 38. With the curing conditions used there was significant nitrite and TSNA accumulation in the cured leaf of the mutant (Fig. 5). *N. plumbaginifolia* is a nornicotine accumulator and the most abundant TSNA was NNN in the mutant. NAT concentration of Wisconsin 38 and the grafted mutant were similar. Nitrite is not translocated between cells in the plant and thus any nitrite in the mutant would have come from microbial reduction of nitrate. Again, this result confirms the essentiality of microbial NRA for TSNA accumulation in curing tobacco.

Figure 5. Nicotine (Nic), nornicotine (Nornic), nitrite-N and TSNA in cured leaf of Wisconsin-38 grafted to N. plumbaginifolia wild type and a nitrate reductase mutant.



3. Leaf Thickness

Because of the significance of moisture for nitrite formation during curing and for alkaloid content and composition, the rate of leaf drying of different cultivars was evaluated. Of the lines studied the dark tobaccos had the greatest leaf thickness at harvest and in the cured leaf (Lei, 2000). Burley tobaccos had the thinnest leaf lamina and the bright tobaccos were intermediate. Leaf density followed a similar pattern with dark tobaccos having the greatest leaf density and burley tobacco the

lowest. However, the rate of water loss was not significantly correlated with thickness or leaf density of these lines. Generally, the burley tobaccos had the least water loss during the first 21 days of air-curing. The dark tobaccos were intermediate and the bright tobaccos had the greatest water loss during the first 21 days of air-curing. The bright tobaccos had the least TSNA accumulation, a result that may support the concept that rapid water loss will reduce TSNA formation, although it must be noted that the bright tobaccos had the lowest levels of alkaloids. Because the curing season was dry, the difference in alkaloid levels among the cultivars was probably the major factor contributing to the difference in the TSNA accumulation.

4. Nitrite scavengers and antibiotics

Nitrite scavengers present in green tobacco and during curing may change TSNA formation by reacting with nitrite, thus reducing the amount of TSNA formed (Schemes 1 and 2). Nitrite scavengers could include amino acids and peptides such as cysteine and glutathione, the antioxidants - ascorbic acid, tocopherols and the polyphenols, especially caffeic acid and lignin (Rundlöf et al, 2000). With many, if not all, of these compounds, the amount present and the location within the cell at the time of nitrite formation are probably most important to TSNA formation. Studies with respect to the status of TSNA formation and the dynamics of the endogenous antioxidants have not been done or are just now in progress.

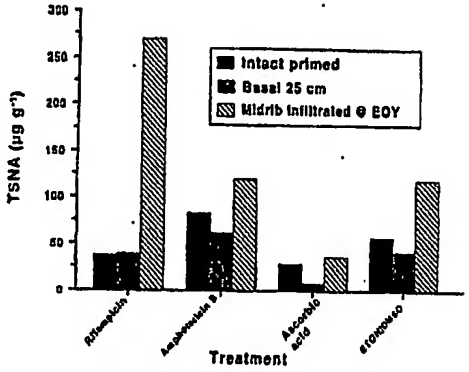
Cui (1998) conducted a series of experiments to evaluate the addition of antibiotics and antioxidants on TSNA accumulation in cured tobacco tissues. In one group of leaves, the butts of the midribs of mature primed burley leaves were placed in solutions of rifampicin, amphotericin B, ascorbic acid, ethanol/dimethyl sulfoxide (as solvent control) or no chemical treatment (control). Approximately 50 ml of solution were absorbed by each leaf. In a second group, the basal 25 cm midrib with adjacent lamina attached and detached were treated by vacuum infiltration of the solution, and in this treatment the tissue gained approximately 25% of their initial weight. In a third group comprising midribs only, the vacuum infiltration of the midrib was done at the end-of-yellowing.

In this series of experiments, all treatments at end-of-yellowing resulted in the highest TSNA accumulation after air-curing for that group (Fig. 6). These results are most likely because of the negative effect from additional water added by the treatment at this critical time. The effect of added moisture for increased microbial activity was apparently greater than any positive effect from chemical treatment. The treatment with ascorbic acid was the only one resulting in significant TSNA reduction in every treatment group compared to other treatments and controls. The lowest TSNA accumulation in the ascorbic acid treatments was found in the basal 25 cm midrib infiltrated with lamina attached. This result may be due to an increased antioxidant uptake by infiltration or a reduced oxidization in the midrib enclosed with lamina

In the cured midrib of the intact primed leaf uptake group, rifampicin and ascorbic acid treatments resulted in lower TSNA accumulation than in the midrib of the primed leaf control (Fig. 7A), however, nitrite concentration in the cured leaf was reduced most significantly only by the ascorbic acid treatment (Fig. 7B).

In the group comprising vacuum infiltration of the basal 25 cm midrib section with adjacent lamina attached, the ascorbic acid infiltration was most effective in reduction of the NNN levels compared to the solvent and primed leaf controls. This result is most likely explained by increased antioxidant uptake by vacuum infiltration. When compared to the solvent control only, the ascorbic acid treatment significantly reduced the TSNA levels (Fig. 8A). The ascorbic acid treatment also had the lowest nitrite accumulation of all chemical treatments but equal to the primed leaf control (Fig. 8B). Similar model experiments have previously been described (Wiernik et al, 1995).

Figure 6. TSNA content in air-cured midrib of chemically treated burley tobacco. Intact were primed leaves fed through the butt of the midrib, basal 25 cm of midrib and lamina were vacuum infiltrated and the midrib alone was infiltrated at the end-of-yellowing.

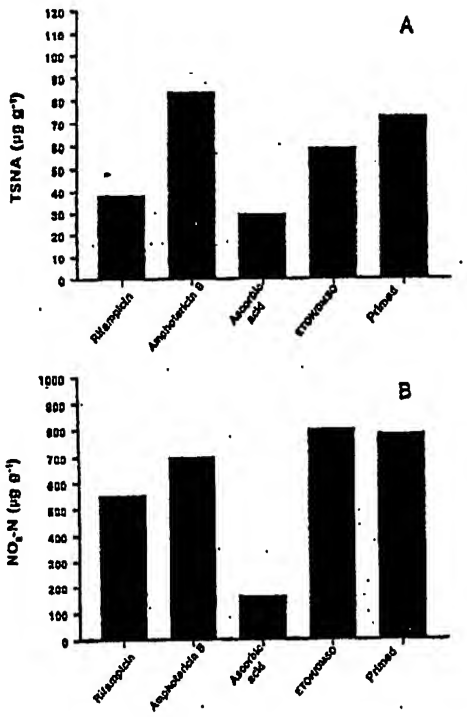


In another series of experiments, Cui (1998) applied the antioxidants, ascorbic acid and a-tocopherol to the leaf surface and physically manipulated the tissue to be cured. Stalk-cut plants and primed leaf were cured in a conventional barn. Greatest reduction (40-65%) of the levels of TSNA and nitrite in lamina and primed leaf was obtained with the addition of ascorbic acid (Fig. 9). Ascorbic acid applied to intact leaves of stalk-cut plants resulted in lower TSNA content in lamina than the stalk-cut controls. a-Tocopherol had no effect on TSNA or nitrite accumulation.

Long et al (1999) misted stalk-cut plants each night for 28 days during the curing process with a 5% ascorbic acid solution. The ascorbic acid treatment increased TSNA levels over 2-fold compared to the non-treated air-cured control. However,

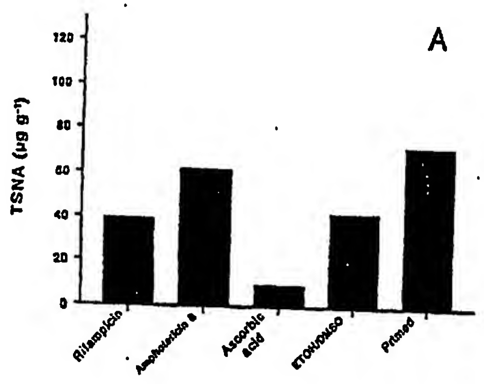
the ascorbic acid treatment decreased the TSNA content by 73% compared to water-misted control.

Figure 7. Total TSNA and nitrite-N accumulation in air-cured midrib of primed leaf treated through the butt end of the midrib of dark tobacco, KY 171.



Results from these types of experiments are not readily repeatable. The reason for the lack of consistent ascorbic acid effect is unknown. However, it would seem that the additional moisture from the application, the effectiveness of uptake by surface application, and unknown factors in the leaf matrix may be involved. Thus, the theory of using nitrite scavengers to reduce TSNA is still valid, but additional investigations are needed to determine how to increase amount of nitrite scavengers on or in the leaf at the critical time.

Figure 8. Total TSNA and nitrite-N accumulation in air-cured midrib of the treated basal 25-cm of the leaf by vacuum infiltration of the treatment solution. Controls were the primed leaf and ethanol/DMSO.



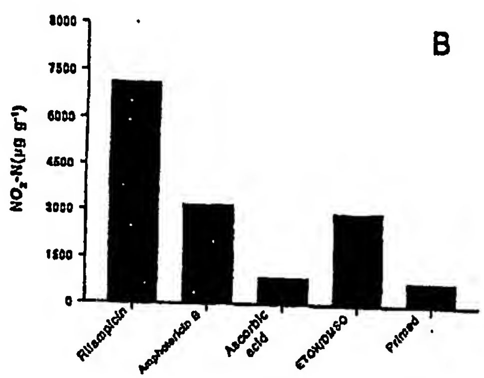
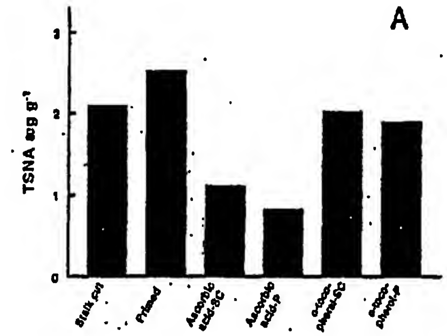
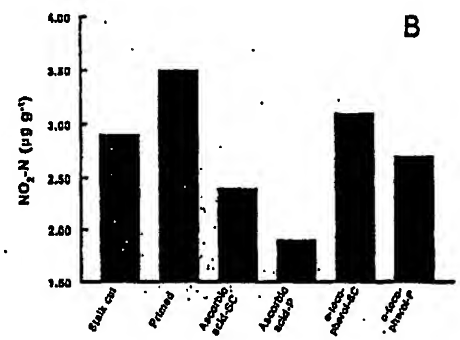


Figure 9. Effect of ascorbic acid and a-tocopherol on TSNA and nitrite accumulation in lamina of dark air-cured tobacco. Treatments were applied at harvest. Primed is primed leaf, SC = stalk-cut and P = primed leaf.

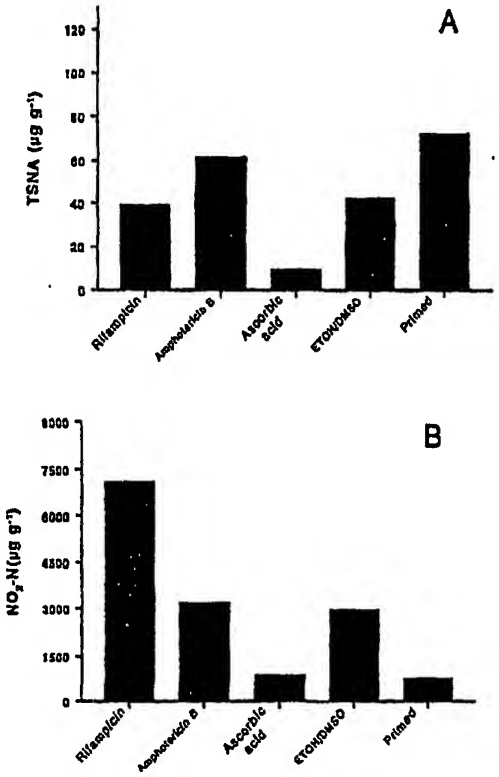




5. Nitrogen fertilization.

Increased nitrogen fertilization generally increases alkaloid and nitrate accumulation in tobacco leaf. Alkaloid accumulation is greatest in the top leaves and lowest in the lower leaves with the lamina containing a higher alkaloid concentration than the midrib portion of the leaf. Nitrate accumulation is generally the opposite of this trend in that greatest nitrate accumulation is found in the bottom leaves and the midrib has a higher concentration than the lamina. With respect to nitrate and alkaloid accumulation, the range of the ratio of that in midrib to that in lamina is 3 to 5 and 3 to 10, respectively. It must be noted that the difference in alkaloid content between leaves from the bottom and top of the plant may be 2 to 3 fold. These differences are as great as many treatment or location effects that are measured. Nitrate levels in the bottom leaves may be 3 to 5 fold higher than in the top leaves. Again, differences between leaves on the same plant are often greater than treatment or location effects.

Figure 8. Total TSNA and nitrite-N accumulation in air-cured midrib of the treated basal 25-cm of the leaf by vacuum infiltration of the treatment solution. Controls were the primed leaf and ethanol/DMSO.



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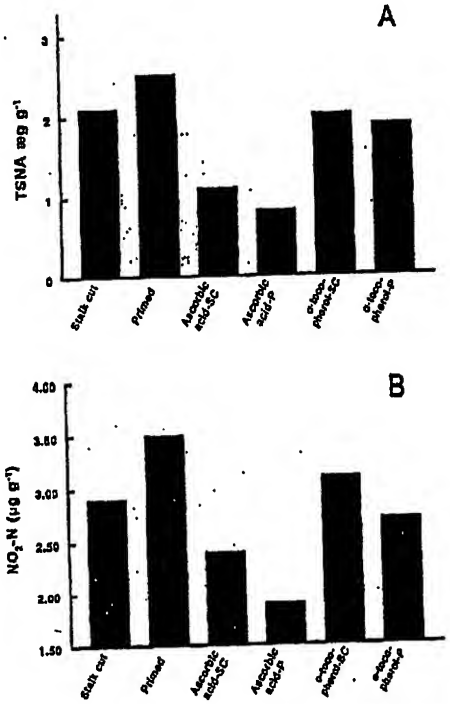

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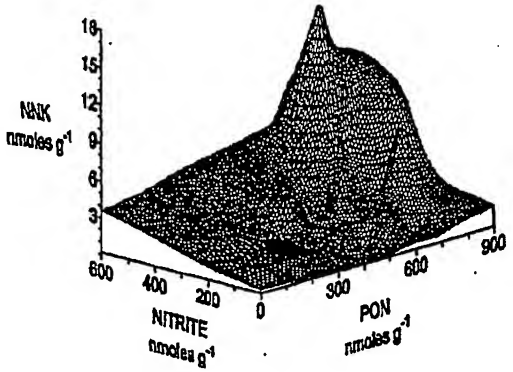
Figure13. Relationships of nitrite and pseudo-oxynicotine (PON) with NNK in air-cured leaf of burley tobacco.



It is quite easy to understand the role of nornicotine, anatabine and anabasine as precursors of the respective nitrosamines NNN, NAT and NAB. Addition of an NO to the secondary amine on the saturated ring is all that is required. Nicotine as the precursor of NNK poses a more complicated mechanistic solution. The process probably requires an oxidation step and then an additional step to form the ring-opened nitrosamine. It would seem that the most accessible intermediate for NNK is pseudo-oxynicotine (PON). Wei (2000) reported that PON levels ranged from 10 to 200 μg g^{-1} in green and cured tobacco. This certainly is sufficient concentration to be considered a precursor for NNK. The distribution of PON closely parallels the distribution of nicotine (Burton et al, 1992). A plot of NNK versus its precursors, pseudo-oxynicotine (PON) and nitrite illustrate most convincingly the case of the dependence of NNK formation on PON (Fig. 13). The molar concentration of both PON and nitrite are in the same range, which results in an easier to interpret relationship than the nornicotine, nitrite and NNN relationship. When both nitrite and PON levels are low in the cured leaf, the concentration of NNK is very low. As the concentration of either PON or nitrite increases, the concentration of NNK increases. Data presented in Figures 12 and 13 support the theory that in the tobacco matrix, TSNA accumulation is dependent on both the secondary amine alkaloid and nitrite concentrations.

CURING ENVIRONMENTS AND STRUCTURES

With knowledge of the mechanisms of the formation of TSNA, it is easy to understand the importance of curing structures and curing conditions on the TSNA accumulation in air-cured tobacco. For many decades the conventional curing environment for air-cured burley or air-cured dark tobacco was that found in a wooden barn with side vents that would allow opening of 1/4 to 1/2 of the sidewall to ventilate the structure during curing. Tobacco should be housed only when dew, rain or other surface moisture has completely dried from the

plant. Burley and dark tobacco are stalk cut and 5 or 6 plants speared onto a tobacco stick, which is hung in the barn. Sticks should be spaced on the rail as far apart as possible for good natural air ventilation. Historically, the spacing is dependent upon the vertical distance between the tier rails, the greater the distance between tiers, the closer the sticks on a rail. With this system the temperature will vary at least 4°C to 5°C from the lower portion of the barn to the upper portion. The relative humidity will vary from 85% in the lower portion of the barn to 50% in the upper portion, when the vents on the sides are closed or there is no wind. If there is some wind, the relative humidity could be from 55% on the windward side to 80% on the lower leeward side of the barn. Combinations of these curing conditions could result in poor air-curing of the tobacco. The following conditions and results are some examples of poor curing from a quality standpoint. It is noteworthy that tobacco cured under these extreme conditions will most likely have low (not detectable to 3 μg g^{-1} in conditions 1, 2 and 3) or high TSNA levels (20 to a few hundred μg g^{-1} in condition 4).

1. Low humidity/high temperature –> a drying and not a cure
2. Low humidity/satisfactory temperature –> piebald or green tobacco
3. Low or high humidity/low temperature –> green tobacco
4. High humidity/high temperature –> house burn, microbial growth and excessive weight loss

Curing structures have changed in the past 10 years and many different versions of "field structures" are now being used. These structures or frames often are just two sticks wide, the sticks cantilevered from the center of the structure and the top and/or sides are covered with black polyethylene film to protect the tobacco from precipitation. Temperatures and relative humidity in these structures may vary significantly from ambient and from those in a conventional barn. Conventional barn temperatures are moderated from the ambient temperature, whereas in outside structures the differences are often magnified (Table 2). Thus, as shown in Table 2, measurements during the curing season of 1999 in Kentucky, USA, demonstrated that outside structures with one or two polyethylene sides had higher temperatures in the tobacco and in the space between the tobacco and the polyethylene covering than in the conventional barn or the structure with no side coverings. For example, with two sides covered, this "gable" area had an average maximum temperature of 42°C during the first 42 days of curing compared to 32°C with no sides covered. The data on hours of relative humidity less than 50% reflect the very dry curing season in 1999.

The temperature and relative humidity within a barn are different among the lower tiers and upper tiers. In one experiment, the average temperature was 23.6, 24.4 and 26.2°C for rails 2, 4 and 6, respectively, during the critical time of

curing for TSNA formation, i.e. days 16 to 35 of the curing process. As the temperature increased, the relative humidity decreased from 72.6, 67.8 to 59.5%, respectively. As a result of these factors, the accumulation of TSNA in the cured leaf decreased from 1.65, 1.62 to 0.91 μg g^{-1} on rails 2, 4 and 6, respectively. In the midrib, the measurements were 0.7, 0.4 and 0.26 μg g^{-1} for rails 2, 4 and 6, respectively. These data are from a year with a dry curing season, and the differences would likely be greater in a normal or moister curing season. Nevertheless, they demonstrate the effect of curing conditions on the TSNA levels in air-cured tobacco.

Table 2. Summary Burley Field Curing Conditions in 1999 at Woodford County Research Farm, Kentucky

Treatment	Avg Max Temp	Avg Min Temp	Avg High RH*	Avg Low RH	Hours RH > 80%	Hours RH < 50%
	°C	°C	%	%	#	#
Ambient	30	12	93	33	281	420
Barn Cure	27	16	89	38	162	371
Outside Structure Measurements in the Tobacco:						
No Sides Covered	29	13	89	33	276	359
Windward Side Covered	31	13	88	32	258	384
Both Sides Covered	34	13	87	31	234	393
Outside Structure Measurements in the Gabel:						
No Sides Covered	32	13	88	32	259	391
Windward Side Covered	38	13	87	29	231	425
Both Sides Covered	42	13	87	27	200	443

**RH= Relative Humidity*

Outside curing structures, often covered with polyethylene to protect the butt ends of the harvested tobacco from rain, are usually only two harvest sticks, 12 plants, wide and tall enough for one tier of plants compared to a conventional barn where frequently there are 9 sticks wide and 4 to 6 tiers of plants high. In 1999, a dry curing season, there were no significant differences between nitrite and TSNA accumulation in cured leaf of several outside curing treatments (Table 3).

In 2000, the tobacco cured in the barn and an outside control structure again had similar TSNA and nitrite levels. However, outside structures modified to increase temperature and relative humidity (to result in a poor curing environment) did increase nitrite and TSNA levels (Table 3). In these treatments, the polyethylene film was wrapped completely around the tobacco and sealed at the soil surface at the time of placing the tobacco in the structure or at the end-of-yellowing phase of curing. In each of these two treatments, one structure had a wind turbine placed in the roof of the structure for ventilation. Air intake was at the ends and bottom of the sealed polyethylene. In both of these treatments, the vented version had lower nitrite and TSNA accumulation compared to the nonvented version.

Table 3. Comparisons among outside curing structures and conventional barn for air-curing burley tobacco.

Curing Structure	NO_2-N μg g^{-1}	TSNA μg g^{-1}
1999		
Barn	1.24	3.5
Outside Structures		
One Side Covered		
sticks 8.9 cm apart	1.10	2.4
sticks 6.7 cm apart	0.097	2.9
Two Sides Covered		
sticks 8.9 cm apart	1.04	3.1
sticks 6.7 cm apart	0.81	2.0
2000		
Barn	12.00	1.5
Outside Structures		
Control, two sides covered	17.55	1.2
Sealed at harvest	467.27	303.5
Sealed at harvest, vented	22.91	4.30
Sealed at EOY	24.74	152.8
Sealed at EOY, vented	31.34	22.2

EOY = end-of-yellowing

These treatments resulted in a great variation in temperature and relative humidity. The structures having the polyethylene sealed at the soil surface at harvest often had temperatures exceeding 40°C during the daytime. The relative humidity reached 100% each night for the first 18 days of the curing process. The corresponding vented treatment did not alter the temperature but the relative humidity was much lower during the daytime, approximately 50% compared to 90% for the nonvented treatment. Treatments of sealing the tobacco at the end-of-yellowing resulted in greater oscillation of daily temperature but the relative humidity was similar to the control treatment.

Increased relative humidity and temperature in the modified structures caused a significant increase in the TSNA levels of the tobacco. However, a "conventional" outside structure is similar to the conventional barn with respect to TSNA accumulation during the curing season.

PLANT MODIFICATION FOR CURING TO REDUCE TSNA LEVELS

Cui (1998) physically modified field-grown mature tobacco at harvest to alter water loss during curing and subsequent TSNA accumulation. Differences in water loss from the lamina was measurable by day 5 of curing with primed leaf treatments being more rapid than intact stalk-cut leaf (Table 4). By day 15 of the

cure, treatments with the midrib attached to the stalk, but split along the long axis, had greater lamina water loss than stalk-cut controls. Water loss from the midrib was also most rapid when the midrib was split along the long axis. After 14 days of curing, 70% of the water had been lost from midribs that had been split and left attached to the stalk. Water loss from primed leaf midrib was most rapid, over 85% loss in 14 days of curing, if the lamina were attached to the split midrib. Splitting the stalk had little effect on water loss of the leaf.

At the end of the curing process, the alkaloid or nitrate content of the cured leaf was similar in all treatments. However, nitrite began accumulating after 21 days in all treatments, but much less accumulation was measured in the primed leaf treatments, especially the primed leaf with the midrib-split. By day 35 of the cure, about 1.5 $\mu g\ g^{-1}$ NO_2-N was measured in the midribs of the primed midrib-split treatment, while 38 $\mu g\ g^{-1}$ NO_2-N was found in the stalk-cut control midribs. Based on the nitrite data, and as expected, there was significantly less TSNA accumulation in the midrib of the primed leaf treatments during curing. At the end of curing, TSNA accumulation was 1.5 to 3.8 $\mu g\ g^{-1}$ in the primed midrib treatments, 7.5 to 8.8 $\mu g\ g^{-1}$ in the midrib-split stalk-cured treatments, but over 12 $\mu g\ g^{-1}$ in the stalk-split or stalk-cured control treatments. Results for the lamina were similar to those for the midrib, and in these experiments, the TSNA accumulation was positively correlated with the nitrite content throughout the curing process, R = 0.95.

Table 4. Percent water loss from lamina and midrib during curing following physical modification of stalk and leaf.

Days	0	3	5	14	21	35
Lamina						
Stalk Cut (control)	0	14	19	78	90	94
Stalk Cut/Split	0	13	31	81	91	94
Stalk Cut, Midrib Split	0	14	32	88	92	95
Stalk Cut/Split, Midrib split	0	18	34	87	90	94
Primed Leaf	0	22	44	83	91	95
Primed Leaf, Midrib Split	0	33	53	85	91	95
Lamina Only	0	31	51	86	92	95
Midrib						
Stalk Cut (control)	0	25	23	40	71	91
Stalk Cut/Split	0	27	29	48	77	96
Stalk Cut, Midrib Split	0	21	39	79	94	97
Stalk Cut/Split, Midrib split	0	27	36	74	93	97
Primed Leaf	0	34	35	55	88	97
Primed Leaf, Midrib Split	0	45	56	86	95	98
Midrib Only		43	55	83	94	97

FUTURE DIRECTIONS FOR MODIFYING ACCUMULATION OF TSNA

To minimize TSNA accumulation all that needs to be done is to inhibit the accumulation of secondary amine alkaloids and/or nitrite. This is a simple solution for a complex problem in air-cured tobacco. In our review of the research on ways to decrease the accumulation of TSNA in air-cured tobacco, it is apparent we know a great deal more about the formation of TSNA during the curing process than was known 10 years ago. We have shown that TSNA accumulation can be decreased, but we have not produced a cured product that has desirable commercial qualities. Because of the nature of the air-curing process and all the potential reactants involved, it is improbable to produce a tobacco that is totally free of TSNA.

Minimizing TSNA accumulation in air-cured tobacco can be separated into a short term and long term approaches. A short term solution is to decrease the accumulation of the secondary amine pyridine alkaloids and, in particular, nornicotine during growth and curing. Decreases of anatabine or anabasine levels in air-cured tobacco are of less importance, since their nitrosated products have little or no significant biological activity. Careful selection of tobacco plants containing very low levels of nornicotine for use in the commercial varieties will result in tobaccos having significantly lower levels of NNN. It is our opinion this is a viable approach for decreasing the amount of NNN in air-cured tobacco and is presently being pursued. The other approach is to inhibit nicotine demethylase activity in the tobacco plant. The major drawback of this approach is that it is longer term and would most likely involve a genetically modified plant. Another long term approach is to inhibit the oxidation of nicotine to PON and subsequently NNK formation during curing. This may be more difficult because of the large amounts of nicotine present and very small amounts of PON required for NNK formation at levels found in tobacco.

The other aspect for decreasing TSNA accumulation is to inhibit or decrease the accumulation of nitrite during curing. There are several approaches that can and have been taken. Perhaps the ideal means is to inhibit only the microflora that has dissimilatory NRA. This selective inhibition would allow other microflora to remain active during the air-curing process. Total inhibition of microbial activity may not be desirable since it may dramatically alter the usability of cured tobacco. Reduced fertilizer nitrogen will decrease alkaloid accumulation but most likely will not affect nitrite availability for TSNA formation during curing. Use of a modified curing process, in which the time for microbial growth is reduced, would most likely result in lowered amounts of nitrite and TSNA in the tobacco. Such a process may, however, be detrimental to the quality and usability of the resultant tobacco. There are no known easy solutions as found for flue-cured tobacco and it will take a cooperative effort of the growers, researchers and manufacturers to produce the highest possible quality air-cured tobacco having the lowest possible TSNA levels.

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THE DETERMINATION OF TOBACCO-SPECIFIC NITROSAMINES IN TOBACCO: A COLLABORATIVE INVESTIGATION OF CURRENT METHODOLOGY.

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ABSTRACT

With the on-going interest in tobacco-specific nitrosamines (TSNAs) in tobacco, analytical methods for TSNAs are constantly being developed and improved. Due to the efforts of the tobacco industry to reduce TSNAs in cured tobacco leaf, many laboratories have expanded their operations to handle the increasing workload. This has lead to a proliferation of procedures and potential confusion when results from different laboratories are compared. So that a standard, validated method throughout the tobacco industry, government agencies and other laboratories may be achieved, the Analytical Methods Committee of the Tobacco Science Research Conference recently has mandated an inter-laboratory collaborative study to establish a method for the analysis of tobacco that is acceptable as a benchmark procedure. Over the past year, 17 laboratories from around the world have participated in the first phase of this collaborative study using at least seven analytical techniques or variations thereof. The methods vary in sample size, extraction technique, sample preparation, internal standards used, instrumental analysis technique and calculation of results. These procedures will be reviewed. Pertinent results will be presented from which conclusions are drawn to merit further study. An outline of the second phase of this collaborative study will be discussed.

INTRODUCTION

The determination of tobacco specific nitrosamines (TSNAs) in tobacco and tobacco smoke has been of interest for many years. Four of the most prevalent N-nitrosamines in tobacco and tobacco smoke and their first reported date of analysis in tobacco are N-nitrosonornicotine (NNN) (1974), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (1978), N-nitrosoanatabine (NAT) (1979) and N-nitrosoanabasine (NAB) (1983) (1, 4, 10, 13, 14). These four N-nitrosamines are usually classified as TSNAs.

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APPENDIX II

Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants

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Key words: ascorbic acid, *Lactuca sativa*, L-gulono-γ-lactone oxidase, *Nicotiana tabacum*, plant metabolic engineering, vitamin C

Abstract

Plants and most animals can synthesize their own L-ascorbic acid (vitamin C), but a mutation in the L-gulono-γ-lactone oxidase gene in the primate lineage makes it necessary for humans to acquire this vital compound from their diet. Despite the fact that plants and animals synthesize ascorbic acid via different pathways, transgenic tobacco and lettuce plants expressing a rat cDNA encoding L-gulono-γ-lactone oxidase accumulated up to seven times more ascorbic acid than untransformed plants. These results demonstrate that basal levels of ascorbic acid in plants can be significantly increased by expressing a single gene from the animal pathway.

Abbreviations: GLDase, L-galactono-γ-lactone dehydrogenase; GLOase, L-gulono-γ-lactone oxidase; L-AsA, L-ascorbic acid; L-GAL, L-galactono-γ-lactone; L-GUL, L-gulono-γ-lactone

Introduction

L-Ascorbic acid (L-AsA, vitamin C) is a powerful antioxidant produced in millimolar concentrations in plants and plays important roles in metabolism and in scavenging free radicals in biological systems. Although the L-AsA pathway in animals is well established, the specific enzymatic steps responsible for L-AsA synthesis in plants have long been controversial [5, 11, 20], and only recently has a generally accepted pathway been proposed for higher plants (Figure 1) [23].

In animals capable of synthesizing L-AsA, the terminal step in the pathway is the oxidation of L-gulono-γ-lactone (L-GUL) by the enzyme L-gulono-γ-lactone oxidase (GLOase, EC 1.1.3.8) [14]. A mutation in the gene encoding this enzyme has rendered primates, including man, incapable of synthesizing L-AsA and therefore dependent on dietary sources for this vitamin.

In plants, early feeding studies suggested that L-galactono-γ-lactone (L-GAL), rather than the L-GUL, serves as the immediate precursor for L-AsA production [8, 12]. Recently, the presence of L-GAL was confirmed in plants and a cauliflower cDNA encoding L-GAL dehydrogenase (GLDase, EC 1.3.2.3) was cloned and expressed in yeast [17].

Even though plants and animals use different biosynthetic pathways to produce L-AsA, it may be possible to metabolically engineer plants to supplement their production of L-AsA using one or more components of the animal pathway. Here we report that tobacco and lettuce plants expressing an animal pathway gene can accumulate up to seven times more L-AsA than control plants.

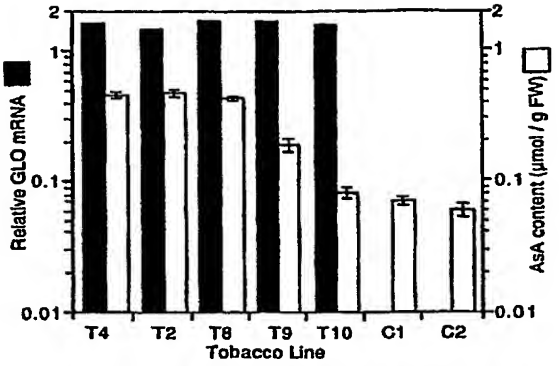

Figure 2. L-AsA content and relative GLOase mRNA expression in leaves of 35S::GLOase transformed (T) and untransformed (C) tobacco lines (error bar indicates standard deviation).

procedure was used throughout the study. The HPLC method was also used to check for L-AsA degradation at different times using both extracted samples and standard solutions. A maximum of 5% degradation in L-AsA peak area was observed during the first 30 min after sample extraction. Therefore, four samples were extracted at a time and they were immediately analyzed to minimize L-AsA degradation. The HPLC procedure is quite rapid with an L-AsA retention time of 3.6 min allowing analysis of four samples in less than 20 min. Three samples from each plant were analyzed to further minimize variations due to L-AsA turnover.

Tissue samples were homogenized in a prechilled mortar with 1:1 (w/v) cold extraction buffer (10% trichloroacetic acid (TCA), 10 mM oxalic acid), and placed on ice for 15 min. The samples were centrifuged at 15000× g for 5 min at 4 °C and the supernatants extracted 3 times with 2 vol of water-saturated ethyl ether to remove TCA and oxalic acid. Standard L-AsA solutions (10 mM) were freshly prepared prior to use.

HPLC analysis of L-AsA was performed using a C-18 reversed-phase column (8 mm × 100 mm). Samples (50 µl) were injected using a Waters WISP 710B autosampler. The solvent system consisted of 80 mM acetate buffer (adjusted to pH 4.0 with glacial acetic acid), 1.0 mM tridecylamine and 15% methanol at a flow rate of 1.3 ml/min. Samples were analyzed with an UV detector at 254 nm. Mean L-AsA levels for each transformed and untransformed individual were calculated and are presented in Figures 2 and 3.

For feeding studies fully expanded young leaves of 35S::GLOase transformed and untransformed tobacco

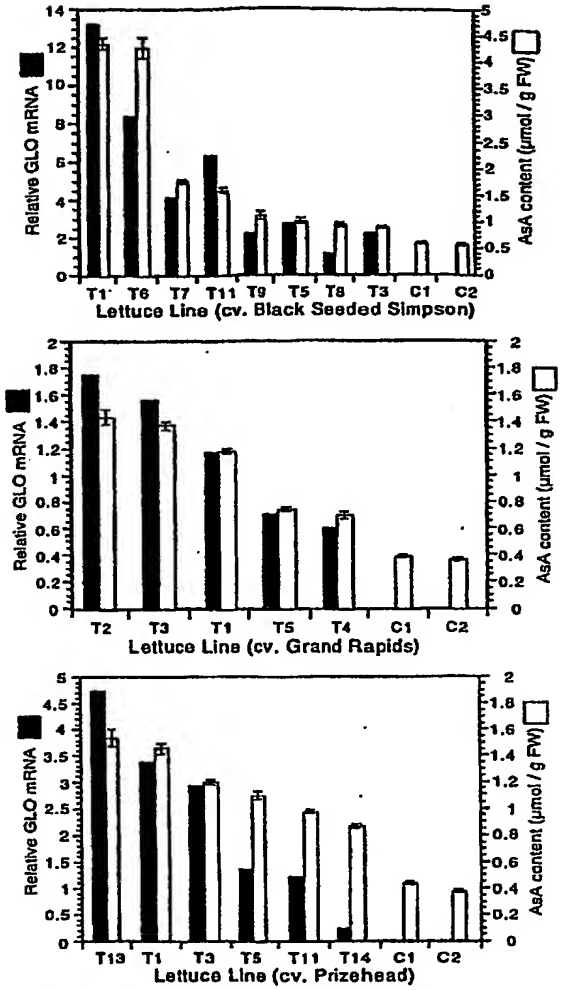

Figure 3. L-AsA content and relative GLO mRNA expression in 35S::GLOase transformed (T) and untransformed (C) leaves from three lettuce cultivars. A, cv. Black Seeded Simpson; B, cv. Grand Rapids; C, cv. Prizehead. Error bar indicates standard deviation.

plants were placed in 125 ml flasks containing deionized water or freshly prepared 30 mM solutions of L-GAL or L-GUL. Leaves were maintained at 22 °C under a 14 h light (40 µmol m^{-2} s^{-1})/10 h dark photoperiod. Leaves were transferred to freshly prepared feeding solutions every 12 h and samples were collected after 72 h for L-AsA analysis by HPLC.

Table 1. Effect of precursor feeding on L-AsA content of transformed (T) and untransformed (C) tobacco leaves

Treatment[a]	L-AsA Content (μ moles per gram FW)[b]			
	T2	T8	C1	C2
L-Galactono-γ-lactone (GAL)	5.80 ±0.876	7.97 ±1.162	3.25 ±0.675	2.85 ±0.487
L-Gulono-γ-lactone (GUL)	1.85 ±0.299	1.94 ±0.251	0.51 ±0.090	0.43 ±0.061
Distilled water	0.14 ±0.006	0.17 ±0.007	0.047 ±0.021	0.041 ±0.018

[a]Leaves were fed 30 mM solutions for 72 h.
[b]Mean of 3 replicates of 2 trials ± standard deviation.

pathway may be a useful approach to nutritional engineering to avoid problems with gene silencing and cosuppression [9, 19, 21]. It will be interesting to see if the introduction of other genes from the animal pathway can influence L-AsA levels in tobacco, lettuce, or other plant species and whether elevated L-AsA levels will be useful in enhancing their tolerance to oxidative stress [5].

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